

A binary plasmid system for shuffling combinatorial antibody libraries

(coexpression/human antibodies/catalytic antibodies)

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ABSTRACT We have used a binary system of replicon-compatible plasmids to test the potential for promiscuous recombination of heavy and light chains within sets of human Fab fragments isolated from combinatorial antibody libraries. Antibody molecules showed a surprising amount of promiscuity in that a particular heavy chain could recombine with multiple light chains with retention of binding to a protein antigen. The degree to which a given heavy chain productively paired with any light chain to bind antigen varied from 43% to 100% and depended strongly on the heavy-chain sequence. Such productive crosses resulted in a set of Fab fragments of similar apparent binding constants, which seemed to differ mainly in the amount of active Fab fragment produced in the bacterial cell. The dominance of the heavy chain in the antibody-antigen interaction was further explored in a set of directed crosses, in which heavy and light chains derived from antigen-specific clones were crossed with uncorrelated heavy and light chains. In these crosses, an Fab fragment retained antigen binding only if it contained a heavy chain from an antigen-specific clone. In no case did the light chain confer detectable affinity when paired with indifferent heavy chains. The surprising promiscuity of heavy chains has ramifications for the evaluation of the diversity of combinatorial libraries made against protein antigens and should allow the combination of one such promiscuous heavy chain with an engineered light chain to form an Fab fragment carrying synthetic cofactors to assist in antibody catalysis.

Random combinatorial antibody libraries have been developed as a means of generating a large number of diverse antibodies (1-3). This method, which provides for random combination of all light and heavy chains derived from a pool of PCR products, has been used to generate a large variety of antibodies derived from mice and humans (4, 5). Such random chain recombination should allow one to go beyond the original diversity of the immune system by isolation of antigen-binding clones with pairings of heavy and light chains that are not present *in vivo*. However, the large number of different chains present in combinatorial libraries prevented a thorough analysis of the ability of given chains to pair productively with complementary chains other than their original partner and to still maintain activity. We now report the development of a binary system of replicon-compatible plasmids which allows efficient chain shuffling in combinatorial antibody libraries. The efficiency of the system is demonstrated for a combinatorial library derived from the bone marrow library of an asymptomatic human immunodeficiency virus (HIV)-positive donor (unpublished data).

MATERIALS AND METHODS

Construction of the Binary Plasmid System. Plasmids pTAC01H and pTC01 are based on the multiple cloning sites of AHC2 and ALC2, a variant of ALC1 (1), and the set of replicon-compatible vectors pFL281 and pFL261 (7). Plasmids pFL261 and pFL281 were generously provided by F. W. Larimer and coworkers. The bacterial host in all experiments was XL1 (Stratagene; *recA1*, *endA1*, *gyrA95*, *thi*, *hsdR17*, *supE44*, *relA1*, *lac* [F' *proAB*, *lacF*, *lacZAM15*, *Tn10(Tet^R)*]). ALC2 and AHC2 were converted into the plasmid form by standard methods (8) and digested with *Sac* I/*Xba* I (pLC2) or *Xho* I/*Spe* I (pHC2). The synthetic linkers (all oligonucleotides obtained from American Synthesis, Pleasanton, CA) 5'-TCGAGGGTCGGTTCGGTCTCTAGACGGTCGGTCGGTCA-3'/5'-CTAGTGACCGACCGACGGTCCTAGAGACCGACCGACCC-3' (for pHC2) and 5'-CGGTTCGGTCGGTCTCTAGAGGGTCGGTTCGGTCT-3'/5'-CTAGAGACCGACCGACCGACCCCTCGAGGACCGACCGACCGACCT-3' (for pLC2) were ligated to the digested, calf intestinal phosphatase-treated vectors. In separate experiments, the multiple cloning sites of pHC2 and pLC2 were transferred into the vectors pFL281 and pFL261 by PCR (9) using Vent polymerase (New England Biolabs) according to the manufacturer's recommendations. The forward primer, 5'-CAAGGAGACAGGATTCATGAATATAC-3', was designed to provide a flush fusion of the *peIB* leader sequence to the ribosome binding sites of the cloning vectors pFL261 and pFL281 via its internal *Bam*HI site (underlined). The reverse primer, 5'-AGGGCGAATTGGATCCCGGGCCGCC-3', was designed to anneal downstream of the region of interest in the parent vector of pHC2/pLC2, pBlueScript SK(-). The resulting light-chain vector, pTC01, was used in this form, whereas the heavy-chain vector was further modified with a sequence encoding an oligohistidine tail to allow purification of Fab fragments by immobilized metal affinity chromatography (10). For this purpose, the synthetic linker 5'-CTAGTCATCATCATCATCATTAAGTATGC-3'/5'-CTAGGTCATCTTAATGATGATGATGATGA-3' was inserted into the *Spe* I site, in effect removing the decapeptide to generate pTAC01H. The expression of Fab fragment in all subsequent cloning experiments was suppressed by adding 1% (wt/vol) glucose to all media and plates.

Construction of Expression Plasmids. Light-chain vector was prepared by digesting pTC01 with *Sac* I and *Xba* I; light-chain insert was obtained by digesting the pComb3-based parent plasmid with the same combination of enzymes and isolating the 0.7 kilobase fragment by electrophoresis in a low-melting agarose gel, followed by *S*-agarose digestion. Ligation was at 16°C overnight under standard conditions using a 3:1 insert/vector molar ratio. Heavy-chain plasmids

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Abbreviations: CDR, complementarity-determining region; HIV, human immunodeficiency virus.

were prepared by digesting pTAC01H with *Xho* I and *Spe* I and ligating it to heavy-chain insert obtained from the pComb3-based parent plasmid (11) by PCR using *Taq* polymerase (Promega) according to the manufacturer's conditions and the primers 5'-CAGGTGCACTCGACGACGTC-TGGG-3' (VH1a) and 5'-GCATGTACTAGTTTGTOTCA-CAGATTGGG-3' (CG12). PCR products were purified by low-melting agarose electrophoresis, digested with *Xho* I and *Spe* I, repurified, and ligated to the prepared heavy-chain vector using a 2:1 insert/vector molar ratio.

Cotransformation of Binary Plasmids. *CaCl*₂-competent XL1 Blue cells were prepared and transformed with ~0.5 µg of each purified plasmid DNA, according to standard methods (8). To select for the presence of both plasmids and the episome, transformants were plated on triple-antibiotic agar plates [carbenicillin (100 µg/ml), chloramphenicol (30 µg/ml), and tetracycline (10 µg/ml) in LB agar (32 g/liter)] containing 1% glucose.

Preparation of Recombinant Fab Fragment. Bacterial cultures for determination of antigen-binding activity were grown (1) in 96-well tissue culture plates (Costar 3596). Two hundred fifty microliters of superbroth (per liter: 10 g of 3-(*N*-morpholino)propanesulfonic acid, 30 g of tryptone, 20 g of yeast extract, pH 7.0 at 25°C) containing chloramphenicol (30 µg/ml), carbenicillin (100 µg/ml) and 1% glucose was added per well and inoculated with a single double-transformant. Plates were incubated with moderate shaking (200 rpm) on a horizontal shaker for 7–9 hr at 37°C, until the OD₅₅₀ was 1–1.5. The cells were collected by centrifugation of the microtiter plate (1500 × g for 30 min at 4°C), the supernatants were discarded, and the cells were resuspended and induced overnight at room temperature in fresh medium containing 1 mM isopropyl β-D-thiogalactopyranoside, but no glucose. Cells were harvested by centrifugation, resuspended in 175 µl of phosphate-buffered saline (10 mM sodium phosphate/160 mM NaCl, pH 7.4 at 25°C) containing phenylmethylsulfonyl fluoride (34 µg/ml) and 1.5% streptomycin sulfate, and lysed by three freeze-thaw cycles between –80°C and 37°C. The crude extracts were partially cleared by centrifugation as above before analysis by antigen-binding ELISA.

Assay and Determination of Relative Affinities. Relative affinities were determined (4) after wells were coated with 0.1 µg of antigen (tetanus toxoid from the Serpitis Clinic, HIB gp120 from American Biotechnologies (Columbia, MD) or SF2 gp120). For each antigen, a negative control extract of

XL1 Blue cotransformed with pT01H and pTAC01H was tested to determine whether other components in *E. coli* had any affinity for the antigens in our assay. Each extract was assayed for binding activity with bovine serum albumin, and albumin-positive clones were considered negative. All possible single-transformants, expressing only one chain were prepared as described for the double-transformants and were found to have no affinity for any of the antigens used; because of the nature of the assay, it could not be determined whether this was due to a lack of binding by the individual chains itself or due to a lack of expression or folding.

RESULTS

Construction of the Binary Plasmid System. A binary plasmid system consisting of two replicon-compatible plasmids was constructed (Fig. 1). The two plasmids feature similar cloning sites including *pelB* leader sequences fused to the ribosome binding sites and the *tac* promoters via *Bam*HI sites (Fig. 2). The heavy-chain vector pTAC01H also encodes a (His)₆ tag to allow purification of the recombinant Fab fragments by immobilized metal affinity chromatography. The presence of both plasmids in the same bacterial cell is selected for by the presence of both antibiotics in the medium. Expression is partially suppressed during growth by addition of glucose and is induced by the addition of isopropyl β-D-thiogalactopyranoside at room temperature. Under these conditions, both plasmids are stable within the cell and support expression of the Fab fragment as assayed by ELISA using goat anti-human α and goat anti-human γ1 antibodies (data not shown).

Direct Coexpression of Heavy and Light Chains Within a Set of HIV gp120/gp160-Binding Antibodies. In the original description (11) of the Fab fragments used in this study, all fragments, derived from the bone marrow of the same asymptomatic HIV donor but panned against HIB gp120, HIB gp160, and SF2 gp120, could be assigned to one of seven groups based on the amino acid sequences of the third complementarity-determining region (CDR3) of their heavy chains (6). From the same library, antibodies to the constrained hypervariable v3 loop-like peptide JSISIGP-GRAPFTYGZC (6) were isolated. For our chain-shuffling experiment, we chose representative members of each of the seven groups (b11; b6; b4, b12, b7, and b21; b3; s8; b1, b14, and b24; b13, b22, B26, b8, b18, b27, B8, B35, and s4) and one loop peptide-binding clone (p35) (6). (B followed by the clone

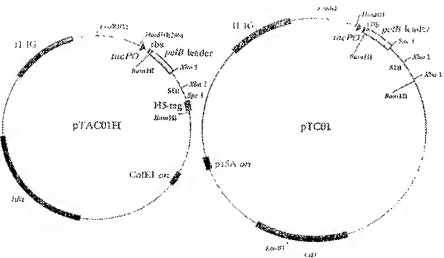


FIG. 1. Plasmid maps of the replicon-compatible chain-shuffling vectors. The two plasmids are very similar in the section containing the promoter and the cloning site. *tacP0*, *tac* promoter; *rbs*, ribosome binding site; *His6*, tag region encoding His₆ tag; *ori*, intergenic region of *l1* phage; *siu*, "stuffer" fragment ready for in-frame replacement by light- or heavy-chain sequences (in pTAC01 or pTAC01H, respectively); *cat*, chloramphenicol acetyltransferase gene; *bla*, β-lactamase gene; *ori*, origin of replication. Maps are drawn approximately to scale.

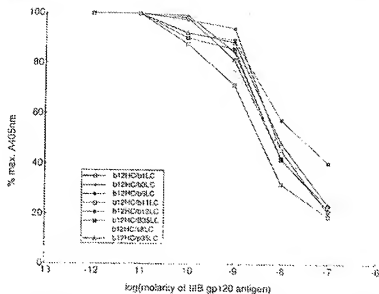


FIG. 4. Affinity of antibody-antigen interaction for b12 heavy chain crosses with light chains from all pannings analyzed by competitive ELISA using soluble IIB gp120 as competing antigen.

B8HC, B35HC, and s4HC. The crosses of the original heavy chains with the original light chains in each case clearly recapitulated binding activity. Minor differences existed between some heavy chains with identical variable-domain sequences—e.g., b4 and b12 (constant domains were not sequenced for any of the constructs). The exception is b8HC, which is identical in its variable domain to b18HC, b27HC, B8HC, B35HC, and s4HC but showed more crossreactivity. Presumably, this was due to differences in expression levels in the cell or differences in the constant-domain sequences. There were clear differences between heavy chains in their tendency to accept different light chains and still bind antigen, but even the least promiscuous heavy chain in the set panned against IIB gp120, b1HC, still did so in 43% of its crosses. On the other side of the spectrum, there were five heavy chains, b11HC, b6HC, b12HC, b7HC, and b8HC, that crossed productively with all light chains in this set. For the heavy chain crosses examined in detail (all of s4HC, B35HC, and B26HC; most of b12HC and b13HC), no significant differences in apparent binding affinity were found between Fab fragments using the same heavy chain but different light chains (Fig. 4).

It is of particular interest that within the original seven groups that were established according to the sequence of the CDR3 of the heavy chains (6) and that are indicated by horizontal and vertical lines in Fig. 3, there is complete promiscuity; i.e., heavy and light chains within these CDR3-determined groups are completely promiscuous with each other. It is equally apparent that between other groups there is a lack of promiscuity—e.g., between b1HC–b24HC and

b13LC–s4LC. In the analysis of these sequence-based groups it does not seem to be important which protein antigen the phage display library was panned against. The exception to this case is the cross of p35HC with all light chains, in that the only cross that bound either to gp120 (SF2 strain) or the original antigen, the loop peptide, was the cross containing the original heavy and light chains (data not shown).

Contrary to our initial hopes, sequence alignments between the most and the least promiscuous heavy chains were not enlightening in that no correlations between promiscuity and amino acid sequence could be established (Fig. 5).

Unlike the heavy chains there are no light chains that will cross productively with all heavy chains nor are there any that are distinguishable from the other light chains by unusually low promiscuity.

Interantigenic Crosses of Heavy and Light Chains. To determine whether conclusions derived from the crosses between high-affinity Fab fragments originating from the same library could be extended to unrelated libraries, we chose a nonrelated y1(x) Fab fragment (P3-13) specific for tetanus toxoid from a different donor for a new set of crosses (clone 3 in ref. 4). Extracts were probed with tetanus toxoid or with gp120 of HIV strain IIB. The data confirm our results from the gp120 cross experiment in that the binding activity toward the antigen was determined by the heavy chain. The heavy chain of clone P3-13 paired with the light chains b4, b12, b21, and b14 to yield an Fab fragment with an affinity for tetanus toxoid; the light chain of P3-13 paired with the heavy chains of b3, b6, b11, and b14 to yield an Fab fragment with an affinity for IIB gp120. None of the light chains originating

FR1	CDR3	FR2	CDR2	FR3	CDR4	FR4
b4, HC	LEEFQGLVYFQSLKALCYGQFTPE	SANNA	WYQVQFQSLQW	LEKAKGKSTETATFVHC	NEISRNGLGDTVYQWRLAQDTATYKAC	QDQVYFQSLQYQWVAGVY
b7, HC	..G..ADVV..AVVV..DA..TP..	NYFTEQF..H	W..WYQVQFQSLQW	..VTFVDAQ..A..VCL..DA..3...X	VS..TIV..SLDQYQWV
b6, HC	LEEFQGLVYFQSLKALCYGQFTPE	SANNA	WYQVQFQSLQW	LEKAKGKSTETATFVHC	NEISRNGLGDTVYQWRLAQDTATYKAC	QDQVYFQSLQYQWVAGVY
b7, HC	..G..ADVV..AVVV..DA..TP..	NYFTEQF..H	W..WYQVQFQSLQW	..VTFVDAQ..A..VCL..DA..3...X	VS..TIV..SLDQYQWV

FIG. 5. Amino acid comparison of least promiscuous heavy chain of gp120-panned clones (b1) and most promiscuous heavy chains (b6 and b7). Matches are indicated by dots. FR, framework region.

from the gp120 binders was able to confer gp120 specificity in combination with the P3-13 heavy chain. Similarly, the P3-13 light chain was unable to generate tetanus toxoid specificity in combination with any of the heavy chains originating from the gp120 binders, confirming the dominance of the heavy chain in the antibody-antigen interaction (13, 14). Interestingly, all three light chains that showed a strong signal against tetanus toxoid (b4, b12, b21) are members of the same group when sorted according to the CDR3 sequences of their original heavy chains. As might be expected from crosses between unrelated libraries, not only was there a lower degree of promiscuity—i.e., chains paired productively with far fewer complementary chains—but the range of apparent affinity constants determined by competition ELISA was much broader (6.3×10^6 – 6.3×10^8 M⁻¹). The replacement of the original P3-13 light chain in the P3-13 Fab fragment with another light chain lowered the affinity of the Fab for tetanus toxoid by a factor of 10–100 (from 6.3×10^8 M⁻¹ to 6.3×10^6 M⁻¹; data not shown). In the crosses of the light chain of P3-13 with all the heavy chains of the HIV pinnings, the productive crosses had similar affinities for IIIB gp120 (2.5×10^6 – 6.3×10^8 M⁻¹), with the exception of b14HC/P3-13L C, whose signal was too weak for a definite determination of the apparent binding constant. These affinities are ~5-fold lower than those of the gp120-specific heavy chains with their original light chains (data not shown).

DISCUSSION

Combinatorial antibody libraries allow the immunochemical analysis of antigen binding to be carried far beyond the limits imposed by the restrictions of the traditional hybridoma technology. Such libraries not only aim to duplicate the diversity of the immune system *in vitro* but may, in combination with synthetic chemical methods (i.e., synthetic libraries), even improve on it in ways not feasible *in vivo* (15). Chain shuffling is yet another maneuver allowed *in vitro* but not *in vivo* which can be expected to help extend antibody diversity beyond that of nature.

The overriding feature of the binary system presented here is its ability to create large numbers (several hundred) of directed crosses between characterized light and heavy chains without the need for recloning individual chains for each cross after the initial vector construction. When used in combination with the phage-display method and biological assays, it allows the rapid analysis of the most interesting subset of the pool of antigen-binding clones by chain shuffling, with the aim of finding biologically or chemically active antibodies. For the set of antigens studied here, we found that most heavy chains will recombine with a number of light chains to yield an antigen-binding Fab fragment. These results have important implications for the diversity of combinatorial antibody libraries. While it is not possible to predict reliably the original *in vivo* combinations of light and heavy chains, due to the surprising promiscuity of individual chains, recombinant antibody libraries may take advantage of the fact that even distantly related Fab fragments against the same antigen can recombine *in vitro* to give chain combinations not found *in vivo*. In fact, after the identification of a certain number of antibodies that have been shown to possess some biological or chemical activity, it may be better to shuffle their individual chains in a directed fashion than to

continue sampling randomly from the same pool of binders. By extension, the promiscuity observed in this system indicates that in libraries constructed using degenerate, chemically synthesized oligonucleotides (15), there should be considerable flexibility in which synthetic heavy chain can pair with which synthetic light chain to generate an antigen-binding Fab fragment.

Ultimately, we hope to use the chain-shuffling system to combine the target specificity of a promiscuous heavy chain with potential cofactors on engineered light chains such as hydrolytically active metals like zinc (6). The diversity of combinatorial libraries coupled with chain shuffling should allow wide exploration of three-dimensional space, thereby solving the problem of how to approximate molecules in the ternary complex of antibody, substrate, and cofactor.

Note Added in Proof. The nucleotide sequences corresponding to the Fab fragments have been deposited with GenBank (L03139–L03180).

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